## REMARKS

Applicants have canceled claims 48-50 without prejudice. Claims 18-20, 23-27, 31-34, 37-43, 46, 47, and 52 are now pending. The Examiner's objection and rejections are addressed below.

**Objection to the Specification**

The Examiner contends that the incorporation by reference of Cwirla et al., Science, 1997, Vol. 276, pp. 1696-99 ("Cwirla") is improper because the mimetic taught by Cwirla is essential to the practice of the claimed invention. The Examiner suggests that the specification be amended to recite that mimetic if that mimetic is "relied upon to overcome any objection, rejection, or other requirements imposed by the Office." Office Action, p. 2. Applicants traverse.

Applicants cite Cwirla merely as one exemplary reference for teaching TPO mimetics. TPO mimetics that can be used in the claimed methods are by no means limited to those disclosed by Cwirla. In fact, Cwirla sets forth in great detail how to screen for and test TPO mimetics. Any other mimetics that are so found can be used in the claimed methods. Thus, contrary to the Examiner's contention, the particular mimetics exemplified in Cwirla are not essential to the implementation of the claims. Further, applicants currently are not relying on Cwirla's mimetics to "overcome any objection, rejection, or other requirements imposed by the Office." No amendment to the specification is hence necessary.

**Rejection Under 35 U.S.C. §112, ¶ 1**

**Part I**

Claims 18, 23, 37 and 48-50 stand rejected for failing to comply with the enablement requirement. The Examiner states that the skilled artisan would need to engage in undue experimentation to implement the claimed methods because the specification fails to disclose a readily available and reproducible source of Retronectin™, a commercially available form of fibronectin, or how to make the compound.

The Examiner is mistaken. Claims 18, 23 and 37 do not recite Retronectin™. Instead, they recite fibronectin generically. Claims 48-50, which do recite Retronectin™, are now canceled in the sole interest of moving the case towards allowance. Thus, the instant rejection should be withdrawn.

**Part II**

Claim 43 stands rejected for failing to comply with the written description requirement. The Examiner contends that applicants have not described a sufficient number of TPO mimetics so that the skilled artisan could envision other mimetics. Applicants traverse.

As acknowledged by the Examiner, the specification cites Cwirla for teaching TPO mimetics. The Examiner is mistaken, however, that a skilled artisan is not in possession of a sufficient number of TPO mimetics. Cwirla discloses a total of 30 peptides that can bind to the TPO receptor and compete with the binding of the natural

TPO (Abstract, first sentence; p. 1697, Table 1; p. 1696, center col.). Thus, a person skilled in the art was in possession of a large enough number of TPO mimetics/agonists, and could have also obtained more by following the detailed methodologies provided by Cwirla (see also discussions *supra*).[1] The written description rejection of claim 43 should be withdrawn.

**Rejection Under 35 U.S.C. § 103(a)**

Claims 18-20, 23-27, 31-34, 37-43, 46, 47, and 52 stand rejected as being obvious over Nolta et al., Blood, 1995, Vol. 86, pp. 101-110 ("Nolta") in view of Young et al., Blood, 1996, Vol. 88, pp. 1619-1631 ("Young") and Kuga et al., Human Gene Therapy, 1997, Vol. 8, pp. 1901-1910 ("Kuga").

According to the Examiner, Nolta teaches pluripotent $CD34^{+}Thy\text{-}1^{+}Lin^{-}$ cells cultured with or without stromal support in the presence of IL-3, IL-6 and SCF, and further transduced with a retroviral vector. He further contends that Nolta teaches that stromal-derived cytokines may be "potentially" used in the absence of stromal cells to increase cycling of quiescent hematopoietic progenitor cells to allow retroviral integration. With regard to Young, the Examiner states that that document teaches (1) pluripotent $CD34^{+}Thy\text{-}1^{+}Lin^{-}$ cells cultured with stromal support in the presence of TPO and a c-kit ligand, and (2) that TPO stimulates division of $CD34^{+}Thy\text{-}1^{+}Lin^{-}$ cells and

[1] Indeed, around of the time of applicants' filing, many other TPO mimetics/agonists were known in the art. See, e.g., Kimura et al., Biochem. And Mol. Biol. Int., 1998, Vol. 44, pp. 1203-1209; De Serres et al., Stem Cells, 1999, Vol. 17, pp. 203-209; and Deng et al. Blood, 1998, Vol. 92, pp. 1981-1988 (attached as Appendices A, B and C).

further enhanced division when combined with IL-3 or a c-kit ligand. With regard to Kuga, the Examiner states that that document teaches (1) transducing $CD34^+$ cells cultured with IL-3/IL-6/SCF and (2) that fibronectin improves transduction efficiency. The Examiner concludes that a skilled person in the art would have been motivated to combine these three documents to arrived at the claimed methods. Applicants traverse.

First, the Examiner is mistaken that Nolta teaches "pluripotent $CD34^+Thy\text{-}1^+Lin^-$ cells" cultured under various conditions. In fact, Nolta's studies are done with $CD34^+$ cells that have **not** been selected for $Thy\text{-}1^+Lin^-$ (p. 102, left col., third full ¶). $CD34^+$ cells are far less enriched for pluripotent stem cells than the $CD34^+Thy\text{-}1^+Lin^-$ population. See, e.g., Tsukamoto, U.S. Patent 5,061,620 ("Tsukamoto"). According to Tsukamoto, less than 10 percent of the $CD34^+$ population in the bone marrow represents true pluripotent stem cells (col. 1, lines 30-55). Therefore the cell population in Nolta is not equivalent to that recited in the claims.

Further, the claimed methods involves the use of the recited cytokines with **no** requirement for stromal support. The inventiveness of the claimed methods lies in part in the discovery that certain combinations of cytokines cause **pluripotent** human hematopoietic **stem** cells (cells that are capable of giving rise to **all** hematopoietic lineages) to proliferate **without** losing their pluripotency, so that the cells can be transduced in a pluripotent stage, rather than a more committed stage. In contrast, Nolta teaches "**loss** of long-lived human progenitors during 3-day in vitro transduction in the **absence** of stromal support" (Abstract, right column, lines 12-14). In view of this

teaching, a person of skill in the art would not have transduced human hematopoietic progenitors in the absence of stromal support. Thus, rather than rendering obvious the instant invention, Nolta teaches **away** from it.

Finally, the claims are directed to methods of transducing human hematopoietic stem cells in the presence of a combination of a mpl ligand, a flt3 ligand and fibronectin. Nowhere does Nolta teach or even suggest this combination of molecules for obtaining genetically modified human pluripotent hematopoietic cells. Rather, Nolta teaches a combination of IL-3, IL-6 and SCF. In addition, Nolta's statement that flk-2 ligand (i.e., flt-3) and some other cytokines are candidate stromal factors does not amount to a teaching. At best, Nolta's statement regarding flt-3 suggests a general approach. That is insufficient. A general approach amounts only to an "obvious-to-try" situation – a standard for obviousness that has been repeatedly rejected.

> An 'obvious-to-try situation' exists when a general disclosure may pique the scientist's interest such that further investigation might be done as a result of the disclosure, but the disclosure itself does not contain a sufficient teaching of how to obtain the desired result, or that the claimed result would be obtained if certain directions were pursued.

*Gillette Co. v. S.C. Johnson & Son, Inc.*, 919 F.2d 720, 725 (Fed.Cir. 1990) (emphasis added; internal citations omitted). Indeed, Nolta provides no data whatsoever to demonstrate that any of those so-called "candidate stromal factors" can facilitate the viral transduction of human hematopoietic stem cells while maintaining their pluripotency.

The secondary references cited by the Examiner, Young and Kuga, do not cure the deficiencies of Nolta. While Young refers to a population of $CD34^+Thy\text{-}1^+Lin^-$ cells and relates to stimulating the expansion of these cells using TPO, LIF and IL-6 from single cells or at limiting dilution (p. 1628, right col., first ¶), or TPO and KL in bulk culture (p.1628, middle ¶ to p. 1630, first ¶), the resulting progeny are only tested for **$CD34^+$**. *See*, e.g., Table 2; Fig. 5. It is unclear whether these progeny cells remained pluripotent as would be indicated by a $Thy\text{-}1^+$ $Lin^-$ phenotype. Nowhere does Young suggest the use of a flt3 ligand, much less the particular combination of a mpl ligand (e.g., TPO), a flt3 ligand and fibronectin. Moreover, like Nolta, the experiments of Young with progenitor cells (from single cells or at limiting dilution) are carried out in the **presence** of stromal cells (p. 1628, right col., middle ¶).[2]

Kuga, the other secondary reference, relates to improving transduction of $CD34^+$ cells pre-incubated with a certain combination of cytokines and fibronectin. Like Nolta, Kuga uses a cell population that is far less enriched for pluripotent stem cells than the $CD34^+Thy\text{-}1^+Lin^-$ cells of the instant claims. Like Nolta and Young, the growth factors used in Kuga do not include the combination of a mpl ligand (TPO), a flt3 ligand and fibronectin. Rather, the combination used in Kuga includes fibronectin, SCF, and IL-6 and/or IL-3. Importantly, assays for successful transduction were performed in CFU-GM, the granulocyte-macrophage (myeloid) progenitor, **not** an **earlier, pluripotent**

[2] Only bulk cultures, which had not been enriched for pluripotent stem cells, were studied in the absence of stromal cells (p. 1628, left col., Discussion, 1st sentence).

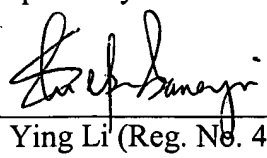
progenitor. See p. 1905, Table 1; p. 1907, Fig. 3. The authors acknowledge that they have demonstrated efficient transduction only in the myeloid progenitors in the $CD34^+$ cell population (p. 1906, Discussion, 1st sentence). Thus, unlike the instant application, Kuga does not demonstrate transduction in true pluripotent hematopoietic stem cells.

In sum, none of the cited references, individually or in combination, teach or even suggest the instant invention. Those references do not use the same cell population as the claimed methods. Further, the primary reference Nolta, which demonstrates the importance of stromal support, teaches away from the claimed methods. Last, the three references use cytokine combinations that are different from each other and from the claimed methods. The Examiner has merely picked and chosen one ingredient from each of the cytokine lists respectively referred to in the references, to arrive at the applicants' unique combination.[3] This pick-and-choose is based on a reading of Nolta, Young and Kuga in hindsight of applicants' invention. Such a hindsight perspective cannot support the asserted obviousness of the present invention.

[3] In fact, none of the references demonstrates that a flt3 ligand, which is required in all of applicants' claims, can help maintain stem cell pluripotency **and** facilitate viral transduction.

For all the above reasons, applicants request that the Examiner withdraw the outstanding rejections and grant allowance to the pending claims.

Respectfully submitted,



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# CONTENTS

Volume 44, Number 6, May 1998



*(Continued on inside back cover)*

*(Continued from outside back cover)*

Vol. 44, No. 6, May 1998 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 1203–1209

# SIGNAL TRANSDUCTION BY THE PEPTIDE WHICH MIMICS THE ACTIVITY OF THROMBOPOIETIN

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**Summary:** Thrombopoietin (TPO) plays a central role in megakaryopoiesis and platelet production. It is a ligand for c-mpl, which is a member of the hematopoietic receptor superfamily. We have recently identified several human c-mpl binding peptides which are distinct from TPO, from phage random peptide libraries. PK1M is one of these peptides and is an agonist of c-mpl which is TPO receptor. We show here that PK1M induces the tyrosine phosphorylation of the Janus kinase 2 (JAK2) and the activation of the signal transducer and activation of transcription 5 (STAT5) in TPO-dependent cells like TPO.

**Keywords:** Thrombopoietin, c-mpl, JAK2, STAT5

## INTRODUCTION

Several years ago, TPO was cloned as the ligand for c-mpl by several groups (1-3) and has been shown to be involved in megakaryopoiesis and platelet production (4-8). c-mpl presents striking homology to the hematopoietic receptor superfamily (9). This superfamily is characterized by 4 conserved cysteine residues at the N-terminus, and the common amino acid motif WSXWS in the extracellular domain (10). Although this superfamily lacks intrinsic kinase activity, a ligand binding induces a rapid increase in the cellular phosphotyrosine content (11). The Janus kinases (JAKs) phosphorylate specific tyrosine residues in the cytoplasmic domain of the cytokine receptor, which act as binding sites that recruit signaling proteins to the receptor (12-14). The signal transducers and activators of transcriptions (STATs) become phosphorylated at particular tyrosine residues, due to the action of the JAKs. This phosphorylation biochemically activates the

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STATs as transcription factors, causing them to oligomerize, translocate to the nucleus, bind to a specific DNA sequence and modulate transcription (12-14). TPO induces the tyrosine phosphorylation of JAK2 and TYK2 and the activation of STAT3 and STAT5 in several cell lines, including mouse megakaryocytes and human platelets (15-26).

Recently we (27) and Cwirla *et al.* (28) have independently detected TPO mimic peptides. We have screened random phage peptide library with a human immunoglobulin fusion protein containing the extracellular domain of human c-Mpl. Several phages were specifically bound to this fusion protein. One peptide derived from it, which were termed PK1M (LQGCTLRAWRAGMC, intramolecular cyclic-form *via* a disulfide bond of cysteines) acted as an agonist of c-Mpl, since it stimulated the proliferation of TPO-dependent cells and the differentiation of mouse bone marrow cells to megakaryocytes. Here we report that the JAK/STAT pathway was activated by PK1M, and we compare the PK1M-induced events with TPO.

## Materials and Methods

**Reagents:** A TPO mimic peptide which we identified and named PK1M was synthesized by Sawady Technology (Tokyo, Japan). The recombinant human TPO was obtained from R & D Systems (Minneapolis, MN). The recombinant mouse interleukin-3 (IL-3) was obtained from Genzyme (Cambridge, MA). Enhanced chemiluminescence (ECL) Western blotting reagents were purchased from Amersham (Buckinghamshire, England).

**Cells and cultures:** Ba/F3 cells were transfected with human *c-mpl* P cDNA (9) as described (27). Ba/F3 cells and the transfectants expressing c-mpl, *i.e.*, BaF/mpl cells, were maintained in RPMI1640 (Nissui, Tokyo, Japan) containing 10% fetal calf serum (FCS; INTERGEN, Purchase, NY), 50 μM β-mercaptoethanol and 5 units/ml of IL-3 at 37℃.

**Immunoprecipitation:** Ba/F3 cells or BaF/mpl cells were IL-3-starved for 16-20 h in RPMI1640 supplemented with 10% FCS. Stimulation was performed with PK1M or TPO at a concentration of $1x10^7$ cells/ml, and the cell lysates were prepared as described by Dorsch *et al.* (16). The cell lysates were incubated at 4℃ with anti-JAK2 rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for several hours and subsequently with protein G agarose (Genzyme) overnight. The immunoprecipitates were boiled with sample buffer (124 mM Tris-HCl, pH 7.2, 2% SDS, 20% glycerol, 2% β-mercaptoethanol) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electroblotted onto nitrocellulose membranes and blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) for 1 h. After being probed with 4G10

antiphosphotyrosine mouse antibody (Upstate Biotechnology Inc., Lake Placid, NY) at room temperature for 1 h in Tris-buffered saline containing 0.5% Tween 20 (TBST), the membranes were incubated with sheep-anti-mouse IgG coupled to alkaline phosphatase (Amersham). After being washed in TBST, the membranes were subjected to the ECL detection system according to the manufacturer's instructions. The same membranes used as above were stripped off the antibody and reprobed with anti-JAK2 rabbit antibody (Santa Cruz Biotechnology) and detected with donkey-anti-rabbit IgG coupled to alkaline phosphatase (Amersham) as described above.

**Electrophoretic mobility shift assay (EMSA):** STAT5 binding site of the β-casein promoter (Santa Cruz Biotechnology, 5'-AGATTTCTAGGAATTCAATCC-3') was used as a probe. The probe was labeled with [γ-$^{32}$P] ATP (Amersham) by T4 polynucleotide kinase (TOYOBO, Osaka, Japan). The stimulation of the cells was carried out as described above. Nuclear extracts were prepared by NP-40 lysis as described by Sadowski and Gilman (29). EMSA was performed using 1.5 μl of nuclear extract in 20 μl of reaction mixture containing 10 mM HEPES, pH7.9, 10 fmol of radiolabeled oligonucleotide, 5% glycerol, 75 mM NaCl, 0.1% NP-40, 1 mg/ml BSA, 1 mM EDTA and 0.5 mg/ml of poly dI-dC. The mixture was incubated at room temperature for 30 min. In the supershift experiments, anti-STAT5B antibody (Santa Cruz Biotechnology) was added, and in the competition experiments, 100-fold molar excesses of unlabeled probe was added. Samples were loaded onto 5 % polyacrylamide gels, then dried and exposed to Hyperfilm ™ (Amersham) overnight.

## Results

**PK1M stimulates the phosphorylation of JAK2:** We previously found that PK1M stimulated the growth of BaF/mpl cells (27). It is known that JAK2 in BaF/mpl cells is tyrosine-phosphorylated in response to TPO (17,18, 25). We examined whether PK1M induces this signal transduction in BaF/mpl cells through c-mpl, as is the case with TPO. Optimal doses for the stimulation of BaF/mpl cells were determined by analyzing the tyrosine phosphorylation of JAK2 in response to increasing doses of PK1M (from 20 ng/ml to 200 μg/ml) or TPO (from 20 pg/ml to 200 ng/ml). The maximal tyrosine phosphorylation of JAK2 was observed after stimulation with 2 μg/ml PK1M (Fig. 1) and 20 ng/ml TPO (data not shown). These tyrosine phosphorylations of JAK2 were not detected in Ba/F3 cells, not expressing c-mpl. These observations suggest that PK1M and TPO use the same signaling pathway *via* c-mpl which is TPO receptor.

**PK1M stimulates the phosphorylation of STAT5B:** Because STAT5 is an early and important substrate of activated JAK2, we next studied whether PK1M induced the

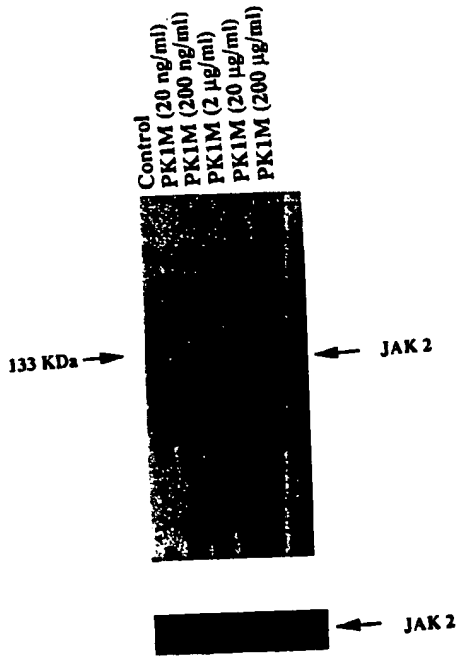


**Fig. 1. Tyrosine phosphorylation of JAK2 kinase in response to PK1M.** BaF/mpl cells were stimulated with PK1M (0-200 μg/ml). The cell lysates were immunoprecipitated with anti-JAK2 antibody and analyzed by SDS-PAGE and Western blotting using 4G10 antiphosphotyrosine antibody (upper panel) or anti-JAK2 antibody (lower panel) as described in Materials and Methods. Molecular weight maker of 133 KDa are indicated.

activation of STAT5B, by EMSA using STAT5 binding site of the β-casein promoter as a probe. Nuclear extracts prepared from PK1M- and TPO-treated BaF/mpl cells possessed DNA binding activity as shown with the probe (Fig. 2). To further examine this result, supershift experiments were performed with anti-STAT5B antibody. The supershift assays demonstrated that the presence of the STAT5B antibody did alter the apparent mobility of the DNA binding complex, indicating the presence of an immunologic STAT5B in PK1M- and TPO-induced extracts. Nuclear extracts prepared from PK1M- and TPO-treated Ba/F3 cells did not reveal DNA binding activity.

## Discussion

Recently we (27) and Cwirla *et al.* (28) detected TPO mimic peptides which stimulate the proliferation of BaF/mpl cells. We have confirmed that PK1M stimulated the

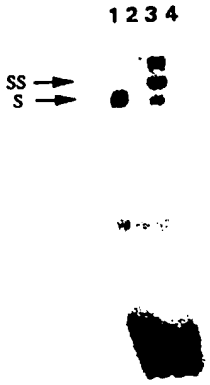


**Fig. 2. Activation of STAT5 in response to PK1M.** BaF/mpl cells were stimulated with 20 μg/ml PK1M. Nuclear extracts of BaF/mpl cells were prepared and analyzed EMSA using STAT5 binding site of the β-casein promoter as a probe. Nuclear extracts were incubated with the $^{32}$P-labeled probe in lane 1, with the $^{32}$P-labeled probe and 100-fold molar excess of unlabeled probe in lane 2, with the $^{32}$P-labeled probe and anti-STAT5B antibody in lane 3, and with only the $^{32}$P-labeled probe in lane 4. Arrow S; Shifted band. Arrow SS; Supershifted band. Similar results were obtained with TPO at 20 ng/ml in BaF/mpl cells (data not shown).

differentiation of mouse bone marrow cells to megakaryocytes by the detection of acetylcholinesterase-positive cells and morphological analysis in a serum-free liquid culture system (27). However, the signal pathway used by a TPO mimic peptide has not been reported. Here we report that the JAK/STAT pathway are activated by a TPO mimic peptide.

The signal pathway of TPO to c-mpl was reported in various cells and human platelets (16-26). It was recently reported that in mouse megakaryocytes, TPO stimulated JAK2 and TYK2 of the JAK family and STAT3 and STAT5 of the STAT family, and that TPO stimulated the same JAKs and STATs in BaF/mpl cells, though it induced a lower level of STAT5 tyrosine phosphorylation in megakaryocytes than in BaF/mpl cells (18). We therefore used BaF/mpl cells to analyze the signal pathway of PK1M. The signal pathway activated by PK1M appear to be identical to those of the natural ligand, TPO. PK1M was observed to induce a pattern of tyrosine phosphorylation of JAK2 and activation of STAT5B indistinguishable from those produced by stimulation with TPO. As TPO

induces other signals such as RAS, MAP kinase *etc.* (30, 31), next we should examine if PK1M can induce these signals as well.

These results further substantiate the future discovery of low molecular compounds which have TPO agonist activity.

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# Immunogenicity of Thrombopoietin Mimetic Peptide GW395058 in BALB/c Mice and New Zealand White Rabbits: Evaluation of the Potential for Thrombopoietin Neutralizing Antibody Production in Man

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Key Words. *Immunogenicity · Thrombopoietin · Mimetic peptide · GW395058 · Mice · Rabbits · Neutralizing antibody*

## ABSTRACT

**Administration of exogenous proteins and peptides as therapeutics carries with it the potential for immune system recognition and the development of neutralizing antibodies to endogenous regulatory proteins. PEGylation of proteins typically reduces their immunogenicity in vivo. GW395058 is a PEGylated peptide thrombopoietin receptor (TPOr) agonist being evaluated for the treatment of chemotherapy-induced thrombocytopenia. Although GW395058 shares no homology with TPO, it does compete with TPO for binding to a common receptor, and a similarity in local structure could result in shared epitopes. Thus GW395058 could elicit TPO-neutralizing antibodies. In this study, we evaluated the immunogenicity of GW395058 in mice, the potential of rabbit antibodies elicited by immunizations with the non-PEGylated parent peptide AF15705 to cross-react with recombinant human (rHu) TPO, and the potential of mouse anti-rHuTPO antibodies elicited by repeated dosing with rHuTPO to cross-react with AF15705. GW395058-dosed mice failed to produce antibodies to AF15705 or rHuTPO. Mouse anti-rHuTPO did not cross-react with AF15705 and rabbit anti-AF15705 antibodies failed to cross-react with rHuTPO. GW395058 caused no immune-mediated lesions in mice, but rHuTPO suppressed megakaryocytopoiesis and caused B-lymphocyte hyperplasia in lymphoid tissues consistent with antigenic stimulation. These data suggest that the potential for an immune response to GW395058 in man would be low.** *Stem Cells 1999;17:203-209*

## INTRODUCTION

Neutropenia and thrombocytopenia are chemotherapy-induced side effects associated with significant morbidity, including spontaneous bleeding. Although recombinant interleukin 11 (IL-11) has been approved for the treatment of chemotherapy-induced thrombocytopenia, its use has been associated with notable side effects [1-3]. Standard practice is to manage thrombocytopenia by administering platelet transfusions, which carry a risk of secondary infection, as well as the eventual production of neutralizing antibodies to platelets. Thrombocytopenia often limits the maximum chemotherapy dose and the dosing regimen that can be administered. Reducing the magnitude of thrombocytopenia might allow administration of higher doses of chemotherapy or dose intensification, which could result in improved disease control.

Recently, the lineage-specific cytokine that regulates platelet production, thrombopoietin (TPO), was identified [4]. Two recombinant forms of human TPO are under investigation: full-length glycosylated recombinant human TPO (rHuTPO) [5] and PEGylated megakaryocyte growth and development factor (PEG-rHuMGDF). PEG-rHuMGDF is a truncated nonglycosylated form of rHuTPO that has been derivitized with polyethylene glycol [6]. In mice, dogs, and primates, rHuTPO and PEG-rHuMGDF promote platelet production and reduce chemotherapy-induced thrombocytopenia [7-14]. In humans, rHuTPO and PEG-rHuMGDF promote platelet production and reduce

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chemotherapy-induced thrombocytopenia [15-19]. Unfortunately, evidence of TPO-neutralizing antibodies in patients participating in cancer and in platelet donor clinical trials forced the discontinuation of PEG-rHuMGDF development [20]. This outcome may affect the future clinical applications of rHuTPO.

Shortly after TPO was isolated, two families of small peptides that bind to the human TPO receptor and compete with the binding of TPO were identified from recombinant peptide libraries. Screening of variant libraries of one of these families yielded a 14-amino acid peptide with high affinity for the TPO receptor. When dimerized, the resulting 28-amino acid peptide AF13948 was equipotent to TPO in cell-based assays [21]. Subsequently, the sequence of AF13948 was modified with amino acid substitution to yield AF15705, which was then PEGylated to produce GW395058 (Fig. 1).

In vitro, GW395058 stimulates HuTPO receptor-transfected BaF3 cells and is equipotent to rHuTPO in stimulating megakaryocyte colony formation [22]. In mice, GW395058 has a long plasma half-life, elevates platelet counts, and accelerates platelet recovery in a mouse myelosuppression model [23]. In dogs, when co-administered with granulocyte colony-stimulating factor, GW395058 has a long plasma half-life and accelerates platelet recovery in a dog myelosuppression model (unpublished results; manuscript in preparation). Preliminary studies of GW395058 in monkeys suggest that repeated dosing with GW395058 does not cause an antibody response to rHuTPO or AF15705 [24].

GW395058 shares no sequence homology with TPO but does compete with TPO for binding to a common receptor site, raising the possibility that a similarity in local structure could result in shared epitopes. Thus, if GW395058 stimulated an antibody response despite being PEGylated, anti-GW395058 antibodies could cross-react with TPO. In this report, we evaluate: A) the immunogenicity of GW395058 in mice after repeated dosing; B) the ability of rabbit antibodies, elicited by immunizations with the non-PEGylated parent peptide AF15705, to cross-react with rHuTPO, and C) the ability of mouse anti-rHuTPO antibodies, elicited by repeated dosing with rHuTPO, to cross-react with AF15705.

## Experimental Procedures

### Materials

rHuTPO was obtained from R&D Systems, Inc., Minneapolis, MN. AF15705 and GW395058 were obtained from the Biotechnology Product Development Division, Glaxo Wellcome Inc., Research Triangle Park, NC. All other chemicals were reagent grade or better.

GW395058 was prepared by derivatizing the two N-termini of the AF15705 linear dimer with 20,000 molecular weight (MW) polyethylene glycol (unpublished results; manuscript in preparation). Derivatization of polyethylene glycol

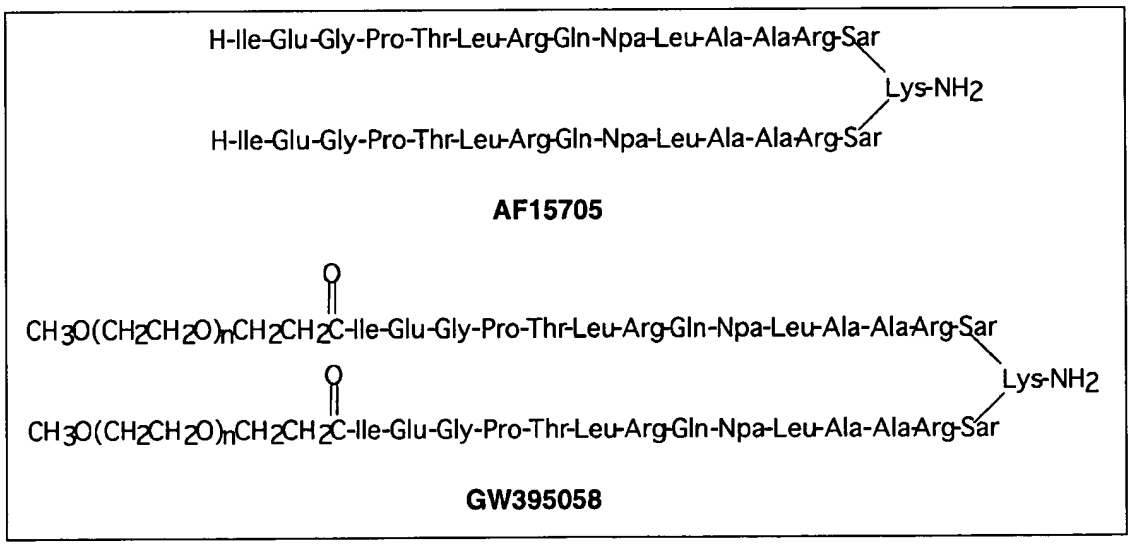


*Figure 1. Amino acid sequences of AF15705 and GW395058. AF15705 is a peptide dimer consisting of two identical amino acid chains of 14 residues linked through their carboxyl termini to the α and ε amino groups of a lysine residue. The sequence of AF13948 (see text) was modified by the replacement of an alanine and a β-alanine residue with sarcosine (Sar) to remove the chiral centers. Tryptophan residues were replaced with napthylalanine (Npa) for ease of synthesis. The two resulting peptide amino termini of AF15705 were conjugated to 20,000 MW polyethylene glycol (PEG) to produce GW395058.*

and its use for preparing protein conjugates has been reviewed elsewhere [25, 26]. The MW of AF15705 and GW395058 are 3,295 and ~43,300, respectively. The amino acid sequences of AF15705 and GW395058 are shown in Figure 1.

For the in vivo experiments described below, dosing solutions of GW395058 or rHuTPO were prepared in phosphate buffered saline. Solution concentrations of GW395058 were determined on the basis of the extinction coefficient of the peptide component. Concentrations or doses expressed in term of total mass of PEGylated peptide would be ~13.5 × greater.

### Animal Handling, Dosing, and Sample Collection

All animal procedures described in this report were approved by the Institutional Animal Care and Use Committee and conducted in accordance with federal guidelines.

Male BALB/c mice (five/group; three dose cycles/group) received s.c. doses of rHuTPO (50 μg/kg; qd × 5/cycle), GW395058 (25 μg/kg; single dose/cycle), or vehicle on Days 1, 43, and 85. Mouse plasma samples for BIAcore™ analysis were collected following the third dosing cycle on day 6 and were stored frozen until use. Blood (15 μl) samples for platelet count determinations were collected from the tail vein after each cycle on day 6 and on days 20, 34, and 41 following the second cycle. Blood was diluted 1:50,000 in isotonic saline prior to analysis. Mouse platelet counts were determined with a Coulter Multisizer II (Coulter Electronics Limited; Luton, Beds, UK).

### Immunological Procedures

New Zealand white rabbits (3 to 5 kg) were initially immunized by s.c. injections with 1.0 mg of an AF15705-bovine thyroglobulin conjugate prepared by using standard glutaraldehyde coupling procedures [27] and then emulsified in Freund's complete adjuvant. Thereafter, rabbits were administered s.c. injections of 0.1 mg antigen in Freund's incomplete adjuvant at approximately two-week intervals for a one- to two-month period. Blood was collected from anesthetized rabbits 7 to 10 days following immunization and the resulting antisera were stored frozen until use.

### Antigenicity Studies

To assess the immunogenicity of GW395058, samples of plasma from BALB/c mice dosed with GW395058 or anti-AF15705 rabbit sera were evaluated by using BIAcore™ analysis. Plasma samples were passed over immobilized rHuTPO or AF15705 to assess the presence or absence of antibodies directed to rHuTPO or AF15705. BIAcore™ technology and its use in characterizing inter-molecular interactions have been described [28, 29]. The BIAcore™ 2000 system, CM5 sensor chips, P-20 surfactant, and the coupling kit which contained N-hydroxysuccinimide, N-ethyl-N′-(3-diethylaminopropyl)-carbodiimide, and ethanolamine hydrochloride (pH 8.5) were purchased from BIAcore™ AB, Uppsala, Sweden. All other chemicals were reagent grade.

The rHuTPO was immobilized to the carboxyl groups of the BIAcore™ CM5 sensor chip hydrogel matrix by activating the matrix with a mixture of 50 mM N-hydroxysuccinimide and 200 mM N-ethyl-N′-(3-diethylaminopropyl)-carbodiimide for 7 min. The rHuTPO (0.33 mg/ml) was diluted 1:10 in 10 mM sodium acetate (pH 5), then injected onto the sensor chip for 3 min at a flow rate of 5 μl/min. Unreacted groups were then deactivated with a 7-min injection of 1 M ethanolamine hydrochloride (pH 8.5). Similarly, AF15705 was immobilized to BIAcore™ sensor chips by activating the chip for 12 min and then applying a peptide solution (prepared by diluting a 1 mg/ml peptide stock 1:5) in 50 mM HEPES (pH 8) containing 1 M NaCl for 20 min. Immobilization was followed by a deactivation step for 12 min. The surfaces of the chips were regenerated after each binding cycle with 100 mM HCl. The BIAcore™ running buffer used for immobilization and binding determinations contained 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% P-20 surfactant.

### Histopathologic Evaluation of Mouse Tissues

Six days after receiving the last doses of rHuTPO, GW395058, or vehicle, mice were euthanized and necropsied. The following tissues were collected, processed by routine histologic methods [30, 31], stained with HE, and examined microscopically by an ACVP-certified veterinary pathologist: adrenal gland (two sections), aorta, brain (three sections), cecum, epididymis (two sections), esophagus, eye (two sections), femur, gallbladder, harderian gland, heart, kidney (two sections), large intestine (two sections), liver (two sections), lung (two sections), lymph nodes (mesenteric and cervical), pancreas, parathyroid gland, pituitary gland, prostate gland, salivary gland, sciatic nerve, seminal vesicle (two sections), skeletal muscle, skin, small intestine (three sections), spinal cord (three sections), spleen, sternum, stifle joint, stomach (two sections), testis (two sections), thymus, thyroid gland, tibia, tongue, trachea, and urinary bladder.

## Results

### Immunoreactivity of BALB/c Mouse Plasma Samples with rHuTPO or AF15705

Plasma samples (dose cycle three) from BALB/c mice that received rHuTPO, GW395058, or vehicle over a three-month period were examined by BIAcore™ analysis for the presence of antibody responses to rHuTPO or AF15705 (Fig. 2).

No antibody binding was observed to either rHuTPO or AF15705 in plasma samples from mice dosed for three cycles with GW395058 as compared to the vehicle control.

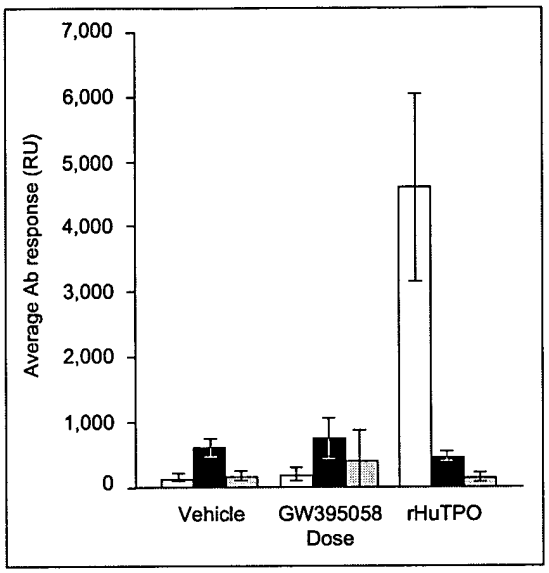


*Figure 2. Antibody responses in mice to rHuTPO or GW395058. Average mouse antibody responses (RU) to rHuTPO, GW395058, or control are shown. Plasma samples were obtained from BALB/c mice (five/group) on post-dose Day 6 (dosing cycle three) following s.c. administration of rHuTPO at 50 μg/kg, qd × 5/cycle (white bar); GW395058 at 25 μg/kg, single dose/cycle (black bar); or vehicle (gray bar).*

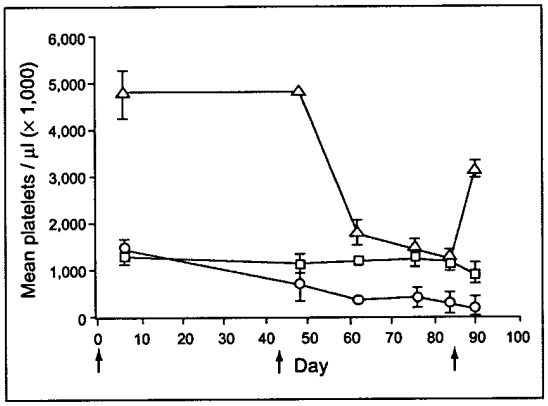


*Figure 3. Platelet responses in BALB/c mice to rHuTPO or GW395058. Average platelet counts in mouse blood samples are shown. Blood samples were obtained from BALB/c mice (five/group; three dose-cycles/group) receiving s.c. doses on Days 1, 43, and 85 (arrows) of rHuTPO at 50 μg/kg, qd × 5/cycle (□); GW395058 at 25 μg/kg, single dose/cycle (Δ); or vehicle (○).*

Mice dosed with rHuTPO showed marked antibody responses to rHuTPO; however, no cross-reactivity with GW395058 was observed in plasma samples from mice dosed with rHuTPO. Mice in the rHuTPO group also showed diminished platelet counts as compared to controls at cycle three with no thrombocytosis in response to rHuTPO administration (Fig. 3). In contrast, mice dosed with GW395058 showed a three- to fourfold elevation in circulating platelet counts after each dose throughout the study.

### Histopathologic Findings in Mice

Light microscopic findings related to treatment with GW395058 or rHuTPO were observed in multiple tissues. Tissue changes related to treatment with GW395058 included: A) increased numbers and size of megakaryocytes in the bone marrow and spleen (Fig. 4); B) occurrence of a few megakaryocytes in the liver and lung; C) reduced erythropoiesis in the bone marrow (Fig. 5), and D) increased erythropoiesis in spleen and liver.

Tissue changes related to treatment with rHuTPO included: A) B-lymphocytic hyperplasia in lymphoid organs (spleen and cervical lymph node), characterized by proliferation of immunoblastic lymphocytes and increased numbers of plasma cells (Fig. 4), and B) reduced numbers of megakaryocytes in bone marrow (Fig. 5).

### Immunoreactivity/Cross-Reactivity of Artificially Generated Antibodies to AF15705 with rHuTPO

The immunoreactivity and cross-reactivity of rabbit anti-AF15705 sera to either AF15705 or rHuTPO were tested by BIAcore™ analysis. The averaged results from 15 individual rabbits are shown in Figure 6. All of the antisera showed strong reactivity with AF15705. None of the antisera showed cross-reactivity with rHuTPO above background control levels.

## Discussion

Recently, patients participating in cancer and in platelet donor clinical trials with PEG-rHuMGDF showed evidence of TPO-neutralizing antibodies, and, as a result, the development of PEG-rHuMGDF was discontinued [20]. The production of TPO-neutralizing antibodies by repeated dosing with PEG-rHuMGDF was unanticipated because prior work has shown that PEGylated proteins often exhibit reduced immunogenicity in vivo [26, 32-35].

GW395058, a PEGylated TPO peptide mimetic, shares no sequence homology with TPO. However, GW395058 does compete with TPO for binding to a common receptor [22, 23]. For this reason, studies were initiated to rule out the possibility that conformational similarities in local structure between GW395058 and TPO at the receptor might lead to the production of TPO-neutralizing antibodies. In preliminary studies of GW395058 immunogenicity, no antibody response was observed to AF15705 or rHuTPO in monkeys dosed repeatedly with GW395058 over a three-month period [24].

Here we show that no antibody response to rHuTPO or AF15705 (the non-PEGylated peptide parent of GW395058)

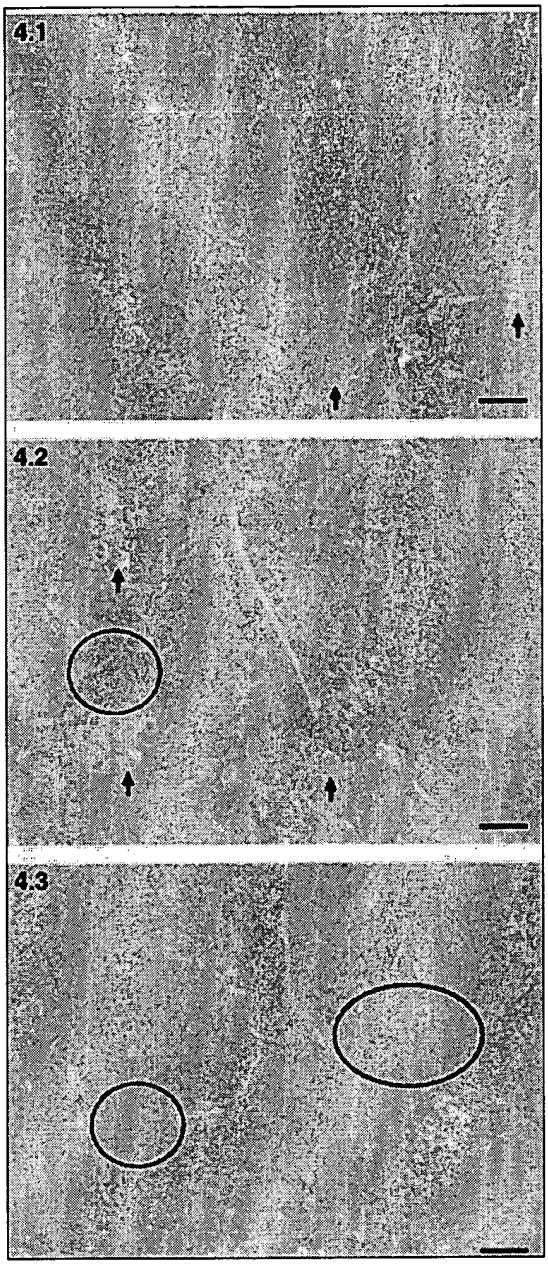


*Figure 4. Photomicrographs of HE-stained spleen sections from mice given three dose cycles of vehicle, GW395058, or rHuTPO. **Panel 1.** Vehicle control. A few small megakaryocytes are present (arrows). Bar = 200 μm. **Panel 2.** GW395058 (25 μg/kg; single dose/cycle). Numerous large megakaryocytes (arrows) and islands of erythropoietic cells (circle) are present. Bar = 200 μm. **Panel 3.** rHuTPO (50 μg/kg, qd × 5/cycle). Megakaryocytes are absent. Bar = 200 μm.*

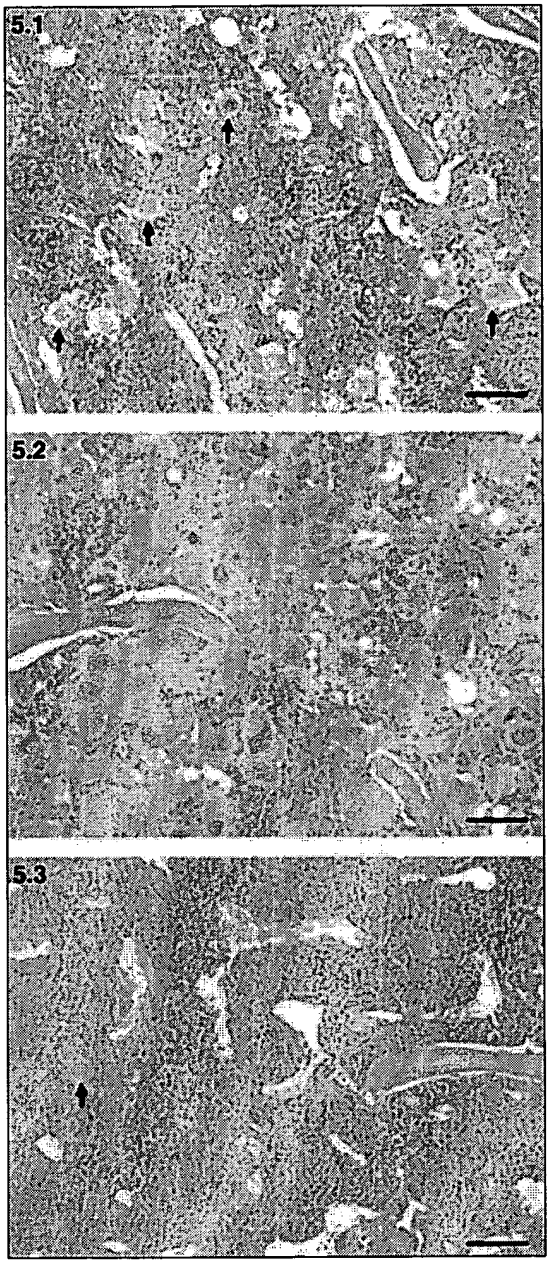


*Figure 5. Photomicrographs of HE-stained spleen sections from mice given three dose cycles of vehicle, GW395058, or rHuTPO. **Panel 1.** Vehicle control. Megakaryocytes are scattered within the marrow (arrows). Bar = 100 μm. **Panel 2.** GW395058 (25 μg/kg; single dose/cycle). Megakaryocytes are numerous and large, consuming much of the marrow space. Both nuclear and cytoplasmic volumes are increased. Bar = 100 μm. **Panel 3.** rHuTPO (50 μg/kg; qd × 5/cycle). Megakaryocytes are scarce. Bar = 100 μm.*

occurred in plasma samples from mice dosed for three cycles with GW395058. In contrast, mice dosed with rHuTPO showed a marked antibody response to rHuTPO, but no murine anti-rHuTPO antibody cross-reactivity with AF15705. Hematological analysis of blood samples from mice dosed with rHuTPO showed diminished platelet counts

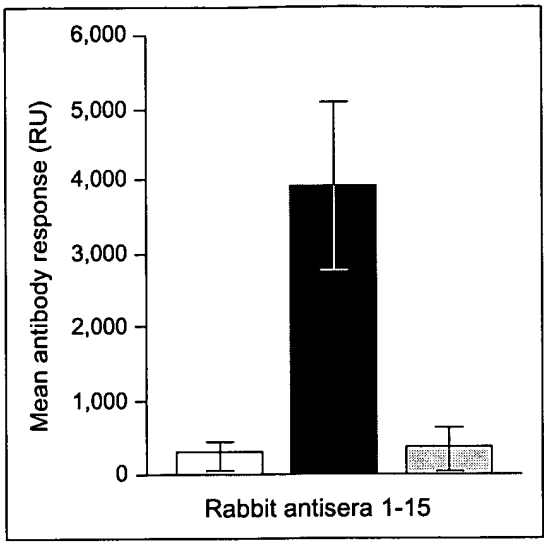


*Figure 6. Cross-reactivity of rabbit anti-AF15705 sera to rHuTPO or GW395058. Average rabbit (n = 15) antibody responses (RU) to rHuTPO (white bar), GW395058 (black bar), or control (gray bar) are shown. Antisera were produced by immunization with an AF15705-bovine thyroglobulin conjugate.*

as compared to controls at cycle three, with no thrombocytosis in response to rHuTPO administration. This observation is consistent with an active anti-rHuTPO antibody response. In contrast, mice dosed with GW395058 showed a three- to fourfold elevation in circulating platelet counts after each dose of the compound throughout the three dosing cycles in this study. This result is consistent with an absence of an antibody response to either GW395058 or endogenous mouse TPO. No histopathologic evidence of antigenic stimulation was observed by microscopic examinations of tissue samples from mice dosed repeatedly with GW395058, while megakaryocytopoiesis in marrow and extramedullary sites was stimulated. In contrast, rHuTPO caused B-lymphocytic hyperplasia in lymphoid tissues, consistent with a response to antigenic stimulation, and suppressed megakaryocytopoiesis in marrow. These pathologic findings suggest that repeated injections of rHuTPO induced antibodies that not only neutralized the injected rHuTPO and prevented it from stimulating megakaryocytopoiesis, but also cross-reacted with endogenous murine TPO to suppress megakaryocytopoiesis.

Rabbit antibodies elicited by immunizations with a thyroglobulin conjugate of the non-PEGylated parent peptide AF15705 failed to cross-react with rHuTPO. While anti-AF15705 sera from all 15 rabbits reacted strongly with AF15705 and GW395058, none of the rabbit antisera cross-reacted with rHuTPO.

Like rHuTPO and PEG-rHuMGDF, GW395058 is a TPO receptor agonist that stimulates platelet production. Unlike rHuTPO and PEG-rHuMGDF, GW395058 is a PEGylated small MW peptide that shares no sequence homology with endogenous TPO. Thus, recognition of GW395058 by the immune surveillance system is minimized. While it is not possible to predict with certainty the immunogenicity of GW395058 in humans from studies in animals, the data presented here suggest that the potential for an immune response to GW395058 in humans will be low. Furthermore, our data suggest that if an immune response to GW395058 were elicited during clinical trials with this compound, such antibodies would be unlikely to cross-react with endogenous human TPO.

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# An Agonist Murine Monoclonal Antibody to the Human c-Mpl Receptor Stimulates Megakaryocytopoiesis

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**Thrombopoietin (TPO) is a hematopoietic growth factor that stimulates megakaryocytopoiesis and platelet production in vivo and promotes the development of identifiable megakaryocytes in vitro. We have developed a murine monoclonal antibody, BAH-1, raised against human megakaryocytic cells, which specifically recognizes the c-Mpl receptor and shows agonist activity by stimulating megakaryocytopoiesis in vitro. BAH-1 antibody specifically binds to platelets and to recombinant c-Mpl with high affinity. Similar to TPO, BAH-1 alone supported the formation of colony-forming unit-megakaryocyte (CFU-MK) colonies. The combination of BAH-1 plus interleukin-3 or of BAH-1 plus human TPO significantly increased the number of human CFU-MK colonies. In addition, BAH-1 monoclonal antibody stimulated the proliferation and maturation of primary bone marrow megakaryocytes in a dynamic heterogeneous liquid culture system. Individual large megakaryocytes as well as small megakaryocytic cells were observed in cultures of CD34$^+$ CD41$^+$ cells in the presence of BAH-1 antibodies. Similar to TPO, BAH-1 antibody induced a significant response of murine immature megakaryocytes as observed by an increase in the detectable numbers of acetylcholinesterase-positive megakaryocytes. No effects of BAH-1 antibody were observed on colony-forming unit-granulocyte-macrophage, burst-forming unit-erythroid, or colony-forming unit-erythroid colonies. In vivo studies showed that BAH-1, alone or in combination with TPO, expands the numbers of megakaryocytic progenitor cells in myelosuppressed mice. This antibody should prove useful in understanding the structure-function aspects of the c-Mpl receptor as well as in evaluating the effects of the sustained activation of this receptor in preclinical models of severe thrombocytopenia.**



THROMBOPOIETIN (TPO) is the primary regulator of physiological platelet production. TPO has dramatic effects on both the proliferation and differentiation of megakaryocytes in vitro and in vivo and is the most potent thrombopoietic agent described to date.[1-13] At therapeutic doses, TPO causes as much as a 10-fold increase in circulating platelet levels.[12] The central role of TPO in megakaryocytopoiesis and thrombopoiesis is further shown by the severe thrombocytopenic phenotype of mice rendered null for the expression of either TPO or its receptor c-Mpl.[14-16] The similarity in the phenotype of the TPO and c-Mpl knockout mice shows that the system is nonredundant and that there is one receptor for TPO and one ligand for c-Mpl. Because of this, it is presumed that binding of TPO to c-Mpl is solely responsible for its activation.

The cell surface receptor (c-Mpl) for TPO is a member of the hematopoietic growth factor receptor superfamily.[17] Extracellular domains of members of this family are typically composed of multiple β-sandwich modules related to the fibronectin type-III immunoglobulin fold, with a characteristic ligand-binding domain formed from two adjacent β-sandwich structures.[18] The mechanism by which TPO activates c-Mpl appears to be similar to that of other hematopoietic growth factors (such as erythropoietin [EPO], growth hormone [GH], prolactin [PRL], and granulocyte colony-stimulating factor [G-CSF]), that are triggered by ligand-induced receptor homodimerization.[19-21] Recently, two families of small peptides that bind to the c-Mpl and compete with the binding of the natural ligand TPO were identified from recombinant peptide libraries.[22] The peptide dimer stimulated the in vitro proliferation and maturation of megakaryocytes from human bone marrow cells.[22]

An antibody having agonist activity that stimulates c-Mpl might serve as an attractive therapeutic option in situations in which a prolonged half-life is needed and in which less frequent administration is desired. Furthermore, mapping the binding site on the c-Mpl receptor of an agonist monoclonal antibody (MoAb) would improve our understanding of the structure-function relationships of this receptor. To these ends, we report on the development of a murine MoAb, termed BAH-1, raised against human megakaryocytic cells that specifically recognizes the c-Mpl receptor, shows agonist activity by stimulating megakaryocytopoiesis in vitro, and also expands the numbers of megakaryocytic progenitor cells in myelosuppressed mice.

## MATERIALS AND METHODS

*Immunization and cell fusion.* BALB/C mice (Jackson Laboratories, Bar Harbor, ME) received repeated injections of $10^7$ CMK cells emulsified in complete Freunds adjuvant according to a previously reported protocol.[23-27] A final booster of $2 \times 10^5$ human primary bone marrow megakaryocytes plus $10^6$ CMK cells was injected 3 to 4 days before the animals were sacrificed.

Spleen cells from the immunized mice were fused with a mouse myeloma cell line (X653). These spleen cells (approximately $1 \times 10^8$) were fused with $2 \times 10^7$ myeloma cells by the addition of 1 mL of 40% polyethylene glycol (Sigma Chemical Corp, St Louis, MO). They were then diluted with 15 mL of Dulbecco's modified Eagle's medium (DMEM), centrifuged, and rediluted into a complete (10% fetal calf serum) selective medium containing hypoxanthine/aminopterin/thymidine at $2 \times 10^6$ cells/mL. The cells were then distributed into 96 wells (100 μL) in hypoxanthine/aminopterin/thymidine medium.[24] After 10 to 16 days, duplicate aliquots of the supernatants were assayed for cell enzyme-linked immunosorbent assay (ELISA) binding. Hybridoma cells from positive testing wells were then transferred to 24 wells

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containing 0.5 mL of hypoxanthine and thymidine medium. After the cells were grown and retested, they were cloned and recloned by limiting dilution into 96-well plates. For the antibody assays, the supernatants from the hybridoma cells were tested directly.

A screening procedure with a whole cell ELISA technique was used to detect antibodies that specifically recognized the human megakaryocytic cell lines CMK, DAMI, Mo7e, and CMS but not T cells, B cells, and monocyte-macrophages.[28,29] After the identification of candidate antimegakaryocyte antibodies, screening for recognition of known megakaryocytic surface structures was done by cross-blocking studies with a panel of antibodies against the integrins GpIb and GpIIb/IIIa.[28,29] One MoAb (BAH-1) that appeared to be specific for megakaryocytes and did not recognize GpIb or GpIIb/IIIa was further characterized. The BAH-1 MoAb was isotyped by using a MoAb isotyping kit (Innogenetics, Antwerp, Belgium), and found to be IgG1 kappa. BAH-1 hybridoma cells were injected intraperitoneally into BALB/C mice primed with pristine, and the antibody-containing ascites fluid was collected 2 to 3 weeks later and affinity-purified as described.[28]

*Growth factors.* Recombinant human interleukin-3 (IL-3) or recombinant murine IL-3, human granulocyte-macrophage colony-stimulating factor (GM-CSF) and human IL-6 were obtained from R&D Systems (Minneapolis, MN). These cytokines were determined to be free of endotoxin contamination. Plateau doses of each factor were determined from dose-response curves for each assay. Recombinant human thrombopoietin (hTPO) and murine thrombopoietin (mTPO; both from Genentech Inc, South San Francisco, CA) were used at 100 ng/mL as determined from dose-response curves in the megakaryocyte progenitor assays (colony formation and liquid cultures). In some experiments, as indicated, we used various dilutions of TPO or IL-3 (10-100 ng/mL) to assess the synergistic effects of both cytokines under conditions of subconcentration or optimum concentration on the megakaryocytic lineage.

*Marrow megakaryocytes.* Human bone marrow was obtained by aspiration from the iliac crests of normal donors who gave their informed consent in a protocol approved by the Deaconess Hospital Institutional Review Board. The marrow was aspirated into preservative-free heparin (Sigma) and separated by centrifugation through Ficoll-Hypaque (Pharmacia Biotech Inc, Piscataway, NJ) at 1,200*g* at room temperature for 30 minutes. After two washes with sterile phosphate-buffered saline (PBS), the cells were resuspended in Iscove's modified Dulbecco's medium with 20% fetal calf serum (FCS), penicillin/streptomycin, and L-glutamine; seeded onto T-75 tissue culture flasks (Corning, Corning, NY); and incubated at 37°C in 5% $CO_2$. After 24 hours, the nonadherent cells were gently removed. Human marrow megakaryocytes were isolated by a method using immunomagnetic beads with antihuman GpIIb/IIIa MoAb as described previously.[11] The cells that rosetted with the immunomagnetic beads were collected with a dynal magnetic particle concentrator (Dynabeads M-450; Dynal Inc, Great Neck, NY) and were washed three times with a megakaryocyte medium, which consisted of $Ca^{2+}$ – $Mg^{2+}$ free PBS containing 13.6 mmol/L-sodium citrate, 1 mmol/L theophylline, 1% bovine serum albumin (BSA), fraction V (Sigma), and 11 mmol/L glucose, adjusted to pH 7.3 and an osmolarity of 290 mOSM/L. After purification, cells were labeled using a MoAb against von Willebrand's factor (MoAb 4F9; AMAC Inc, Westbrook, ME), and more than 95% of the cells were stained. Twenty milliliters of bone marrow aspirate generally yielded about $1 \times 10^5$ megakaryocytes. Contaminating cells (1%-5%) were essentially monocytes and macrophages. Cells were cultured in RPMI-1640 supplemented with 2% platelet poor plasma (PPP)[11] at 37°C in a 5% $CO_2$ fully humidified atmosphere for 24 hours. Monocytes and macrophages were identified by morphology after May-Grunwald-Giemsa staining and by positive antibody staining using a MoAb directed against CD14 (monocytes), CD15 (granulocytes), CD16 (IgG Fc receptor-natural killer cells, granulocytes, and macrophages; AMAC Inc). These analyses indicated that the maximum potential degree of contamination of bone marrow megakaryocytes after 24 hours was about 5% to 10%.

*Isolation of $CD34^+$ cells by the immunomagnetic bead technique.* $CD34^+$ cells were isolated as described.[12] Cells were first incubated at 4°C for 30 minutes with the $CD34^+$ MoAb at a concentration of 10 mg/mL, and then with paramagnetic beads coupled with goat antibody to mouse IgG (Dynabeads M-450; Dynal) with a bead-to-target cell ratio of 5:1. $CD34^+$ cells were isolated by magnetic separation and detached from the beads by chymopapain treatment (Sigma; 130 U/mL for 10 minutes), which allows for the collection of $CD34^+$ cells free of beads.

*Megakaryocyte progenitor assay.* Low-density bone marrow cells were cultured in a semisolid medium by using the plasma clot technique.[11] The medium consisted of RPMI-1640, 1% deionized BSA, 20 mg/mL asparagine, 28 mg/mL $CaCl_2$, 10% PPP, and $2.5 \times 10^5$ nonadherent bone marrow cells in the absence or presence of various dilutions of the MoAb BAH-1. Ten percent citrated bovine plasma (GIBCO, Gaithersburg, MD) was added as the last product. The PPP and citrated bovine plasma used in these cultures were assayed and determined to be devoid of any detectable IL-6 or endogenous transforming growth factor-β (TGF-β) by specific immunoenzymatic assays for IL-6 and TGF-β, respectively (R & D Systems). Cultures were incubated for 12 days at 37°C in duplicate. Quantitation of colonies was performed by an ABC labeling kit (Vector Laboratories, Burlingame, CA) by using anti-GpIIIa antibodies. Each dish was entirely scanned under a microscope at day 12 of culture, and each cluster of three or more megakaryocytes was scored as a colony. For the limiting dilution experiments, $CD34^+$ $CD41^+$ cells were directly sorted into 96-well tissue culture plates and the numbers of megakaryocytes were identified by a GpIIb/IIIa cell-based ELISA.[11]

*Human megakaryocytic cell lines.* The megakaryoblastic cell lines CMK,[30,31] DAMI, and Mo7e were provided by Dr T. Sato (Chiba University, Chiba, Japan), Dr S. Greenberg (Brigham and Women's Hospital, Boston, MA) and Dr J. Hoxie (University of Pennsylvania, Philadelphia, PA), respectively. Each cell line was cultured as previously described.[32] Jurkat T cells were obtained from the American Type Tissue Collection and maintained in liquid culture according to the specifications in the catalog.

*Murine megakaryocyte assay.* To assess megakaryocytic differentiating activity, a single megakaryocyte growth assay was used.[31] Single cell populations from bone marrow were prepared from the femurs of normal C57/BL6 mice. This preparation was performed by flushing the bones with DMEM containing 10% FCS. Immature megakaryocyte populations were obtained in 1.07 to 1.085 $g/cm^{-3}$ fractions, from a suspension of single bone marrow cells separated in a Percoll gradient. The fractionated cells were cultured in 10% FCS in DMEM for 5 days at 37°C in a 10% $CO_2$ humidified incubator. This procedure was performed in the presence of titrated doses of growth factors. Cultures were dried and stained for acetylcholinesterase. Growth and maturation of immature megakaryocytes were quantitated by assessing the number of single large megakaryocytes detected by light microscopy.

*Mice treatments and assays.* For the myelosuppression experiments, 6- to 9-week-old female BALB/C mice received a single intraperitoneal injection of 1.2 mg carboplatin and 350 cGy whole-body $^{137}Cs$ irradiation (Gammacell 40 irradiator; Atomic Energy of Canada Radiochemical Co, Kanata, Canada) on day 0. The following day, the mice were begun on daily intraperitoneal injections of vehicle (20 mmol/L Tris, pH 8.1/0.9% NaCl/0.25% rabbit serum albumin), recombinant murine TPO (40 kU/mouse/d) in vehicle, purified BAH-1 antibody (5 mg/mouse), or both TPO (40 kU) and BAH-1 antibody (5 mg/mouse).

Animals were sacrificed 13 to 14 days after the initiation of treatment, which was 2 to 3 days before the onset of platelet recovery in the TPO-treated mice.

After sacrifice by cervical dislocation, the femurs of each study

mouse were harvested and single-cell suspensions were prepared by using standard techniques.[33,34] From 0.5 to 2 × $10^5$ cells/mL were plated for megakaryocytic colony formation (CFU-MK) using 20 ng/mL murine IL-3 plus 7 ng/mL murine TPO in agar as previously described.[8] As these cultures contained optimal levels of IL-3, granulocyte-macrophage colonies (CFU-GM) were also enumerated. Assays for erythroid bursts (BFU-E) were performed using recombinant human EPO as previously described.[8] Late erythroid progenitors (CFU-E) were assayed in a plasma clot in the presence of 0.5 U/mL EPO.[35] Each assay was performed in duplicate.

*Flow cytometric analysis of surface protein expression.* To detect the potential surface binding proteins that bind BAH-1, we used flow cytometric analysis (FACS staining). Cells were washed with sterile PBS, and 1 × $10^6$ cells were resuspended in 0.1 mL of PBS. Cells were incubated with 10 mL of the BAH-1 MoAb or GpIIIa antibodies, mouse IgG as a control (Immunotech Inc, Westbrook, ME) or PBS at 4°C for 20 minutes. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or goat antirabbit IgG was added at a final dilution of 1:500 and followed by incubation for 20 minutes at 4°C. Cells were washed twice and resuspended in 0.5 mL of 1% (vol/vol) paraformaldehyde in PBS. Cells were then analyzed by flow cytometry.

*Immunoprecipitation and Western immunoblotting.* CMK cells (2 × $10^6$/mL) were serum-starved for 4 to 5 hours in RPMI-1640 medium. Cells were centrifuged, then resuspended at $10^7$ cells/mL in the RPMI-1640. Cells (20 × $10^6$/precipitation) were placed on ice and lysed by the addition of 1/3 volumes of 3× lysis buffer (40 mmol/L Tris-HCl, pH 7.4/2 mmol/L $MgCl_2$/2 mmol/L $CaCl_2$/20% glycerol/2% NP-40/2 mmol/L $Na_3VO_4$/20 mg/mL leupeptin/20 mg/mL aprotinin/4 mmol/L phenylmethyl sulfonyl fluoride [PMSF]). Lysates were centrifuged at 10,000*g* for 15 minutes. The MoAbs BAH-1 or Mpl-R (Genzyme, Cambridge, MA) were added to the supernatant at 5 mg/precipitation. Tubes were incubated by rocking at 5°C for 3 hours, and then 40 mL of 1:1 Protein G-Sepharose (Pierce, Rockford, IL) were added. After 1 and a half hours, lysates were washed three times with 1× lysis buffer. Sodium dodecyl sulfate (SDS) sample buffer was added to the washed beads and samples were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% acrylamide). SDS polyacrylamide gels were electrophoretically transferred onto nitrocellulose membranes (BioRad, Hercules, CA). The membranes were blocked with 4% BSA in PBS/0.1% Tween 20 (PBST) and then incubated with the BAH-1 MoAb (0.2 mg/mL) for 1 and a half hours or with Mpl-R MoAb (0.2 mg/mL). Membranes were then washed three times in PBST and incubated for 45 minutes in horseradish peroxidase-linked secondary antibody (Amersham Corp, Arlington Heights, IL) diluted in PBST. Transfers were washed three times in PBST and developed by the enhanced chemiluminescence (ECL) method (Amersham).

*Binding of BAH-1 to platelets and c-Mpl.* Platelet rich plasma was prepared by centrifugation of citrated whole blood at 400*g* for 5 minutes. Binding studies were conducted within 3 hours of collection. $^{125}$I-BAH-1 was prepared by indirect iodination and yielded a specific activity of 15 to 50 mCi/mg protein. $^{125}$I-BAH-1 (100 pmol/L) was incubated with 4 × $10^7$ platelets in plasma at 37°C for 30 minutes with varying concentrations of unlabeled BAH-1 in triplicate. The reaction mixture was overlayed on 1 mL of Hank's balanced salt solution containing 0.5% BSA and 20% sucrose, then microcentrifuged at 13,500 rpm for 5 minutes. The supernatants were aspirated, tube bottoms containing the cell pellets were cut off, and platelet-associated radioactivity was determined.

The binding of BAH-1 to recombinant c-Mpl was determined by using a solid phase assay as described.[36] Ninety-six–well immunoplates were coated with 50 mL of rabbit antihuman IgG Fc (Jackson Labs; 2 mg/mL in carbonate buffer, pH 9.6) overnight at 4°C. After blocking for 1 hour with PBS containing 0.5% BSA, the plates were incubated with conditioned media from the Mpl-IgG transfected 293 cells. Plates were subsequently washed three times with PBS containing 0.5% BSA and 0.05% Tween 20 (assay buffer) and $^{125}$I-BAH-1 (100 pmol/L) was added with varying concentrations of unlabeled BAH-1. After 1 hour, the plates were washed five times with assay buffer and bound $^{125}$I-BAH-1 was eluted with 4% SDS in 0.1 mol/L acetic acid and then quantitated in a gamma counter.

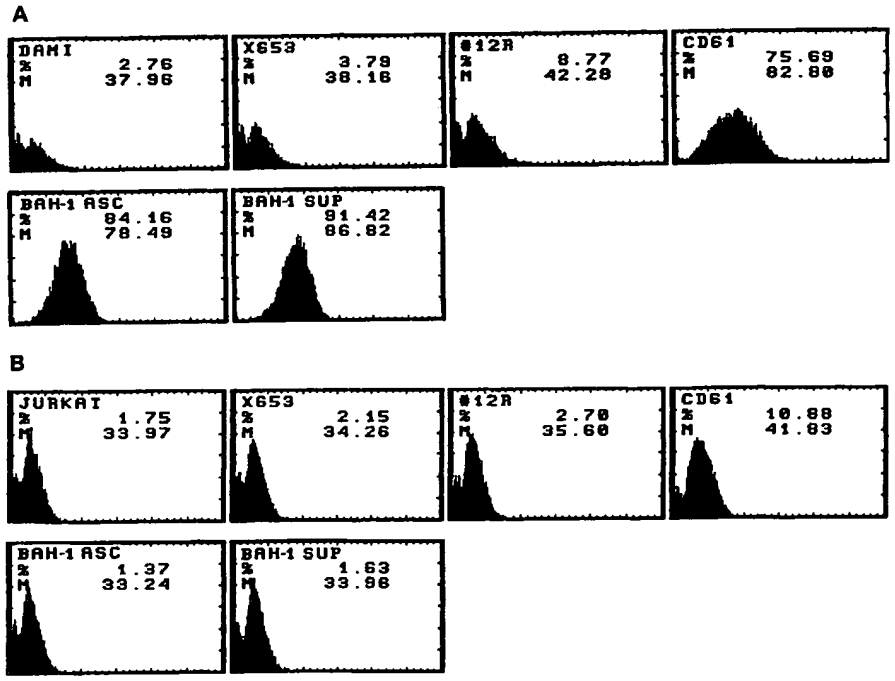



**Fig 1. Flow cytometric analysis of BAH-1 surface protein expression in a DAMI megakaryocytic cell line. Antigen expression was evaluated by FACS staining using BAH-1 and Mpl-R MoAbs (commercially available from Genzyme, MA), CD61 or control MoAb on DAMI (A) and Jurkat T cells (B). X653, supernatant from the mouse myeloma cell line used in cell fusion; 12R, a control nonrelevant MoAb (1:1,000); CD61, a positive control for megakaryocytic cells; BAH-1 Asc, ascites of BAH-1 (1:1,000 dilution); BAH-1 Sup, BAH-1 supernatant (1:1,000 dilution).**

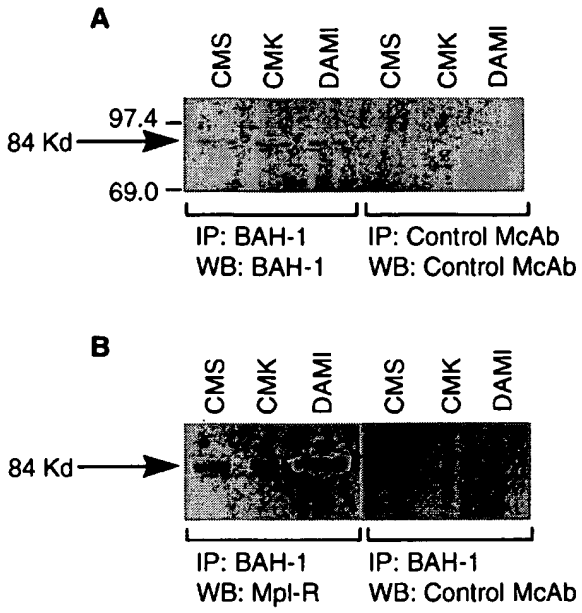



**Fig 2. Immunoprecipitation of Mpl-R protein. CMK cell extracts were immunoprecipitated with BAH-1 antibodies and subjected to SDS-PAGE followed by Western blotting using control MoAb or BAH-1 antibodies (A) or Mpl-R antibodies (B) as described in the Materials and Methods section. Arrow indicates the position of the c-Mpl. The reactive proteins were detected by using the ECL system (Amersham).**

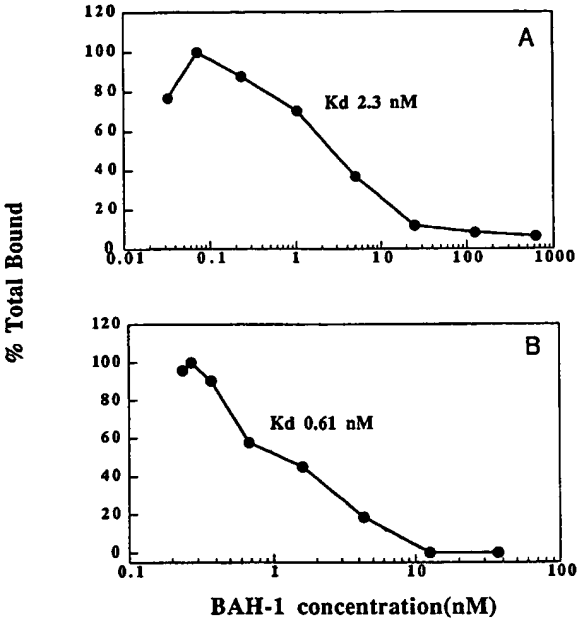



**Fig 3. Binding of BAH-1 to human platelets and recombinant c-Mpl. Equilibrium binding studies for $^{125}$I-BAH-1 were performed on platelets (A) and c-Mpl-IgG fusion protein (B) as described in the Materials and Methods. The kd was determined as described.[40]**

*Statistical analysis.* The results were expressed as the mean ± standard error of the mean (SEM) of data obtained from three or more experiments performed in triplicate. Statistical significance was determined using the Student's *t*-test.

## RESULTS

*Characterization of the MoAb BAH-1.* We derived one murine MoAb (BAH-1), which appeared to specifically interact with the human megakaryocytic cell line DAMI. Immunofluorescence staining showed that more than 90% of the DAMI cells stained positive with BAH-1 MoAb. Similar results were obtained with other megakaryoblastic cell lines, such as CMK, CMS, and Mo7e (data not shown). No staining was observed when Jurkat T cells were used (Fig 1). In addition, this antibody did not recognize the major megakaryocytic glycoproteins Ib or IIb/IIIa (data not shown). Immunoprecipitation and Western blot analysis showed that this MoAb reacted specifically with the c-Mpl protein (Fig 2). BAH-1 immunoprecipitated an 84-kD protein from CMK cell lysates that cross-reacted with a known c-Mpl antibody (Mpl-R).

We also determined the ability of BAH-1 to bind to platelets and recombinant c-Mpl (Fig 3). In equilibrium binding studies, a kd of 2.3 nmol/L was determined for BAH-1 binding to platelets. This compares to a kd of 200 pmol/L for TPO.[36] By using a recombinant truncated version of c-Mpl, which consists of the extracellular domain of c-Mpl fused to an Fc domain of IgG,[36] we found that BAH-1 also bound specifically to purified c-Mpl with a kd of 0.61 nmol/L. These results show that BAH-1 specifically binds c-Mpl with high affinity. Interestingly, compe-

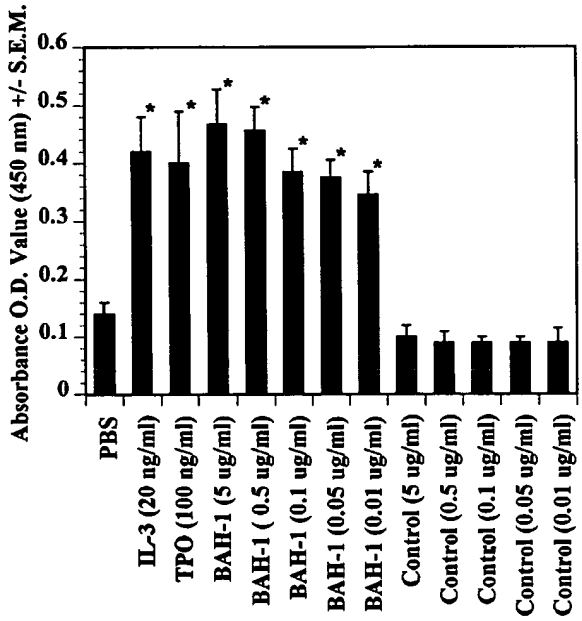



**Fig 4. Effects of TPO, IL-3, and BAH-1 MoAb on megakaryocyte differentiation of CD34$^+$ cells. CD34$^+$ cells (1 × 10$^4$/mL) were cultured in 500 mL of serum-free culture medium. After 10 days, the increase in megakaryocytes was determined by ELISA using anti-GpIIb/IIIa antibodies. Results represent the mean optical density (OD) ± SEM of three independent experiments. *Statistically significant compared with PBS ($P < .05$).**

tition experiments showed that BAH-1 did not inhibit TPO binding to c-Mpl, suggesting that TPO and BAH-1 bind to different sites on the c-Mpl receptor (data not shown).

*Functional characterization of BAH-1 MoAb.* The effects of BAH-1 MoAb on megakaryocytopoiesis were evaluated in liquid suspension and fibrin clot megakaryocytopoiesis assays. For the liquid suspension assay, purified $CD34^+$ cells ($5 \times 10^4$/mL) were cultured in serum-free medium in the presence of various concentrations of BAH-1 for 10 days. As shown in Fig 4, BAH-1 stimulated the production of megakaryocytes in these cultures. A plateau was reached at 100 ng/mL of BAH-1, a level of stimulation that is similar to that caused by 100 ng/mL of TPO (Fig 4). Similarly, in the fibrin clot system BAH-1 also stimulated the production of CFU-MK progenitors (Fig 5A and B). In combination with IL-3, BAH-1 acted additively. No effects were observed on CFU-GM, BFU-E, or CFU-E colonies when BAH-1 antibody was used in various concentrations.

We also determined the ability of BAH-1 to directly stimulate the proliferation of early megakaryocyte progenitors. In limiting dilution experiments, $CD34^+$ $CD41^+$ cells were plated at a concentration of 1 to 50 cells (100 mL volume) in the presence of TPO, IL-3, or BAH-1 alone or in combination. After 5 days in culture, overall expansion was determined and megakaryocytes quantitated by staining with an anti-IIb/IIIa antibody. As shown in Fig 6, the effect of BAH-1 was similar to that of TPO. The percentage of positive megakaryocytes in each well was

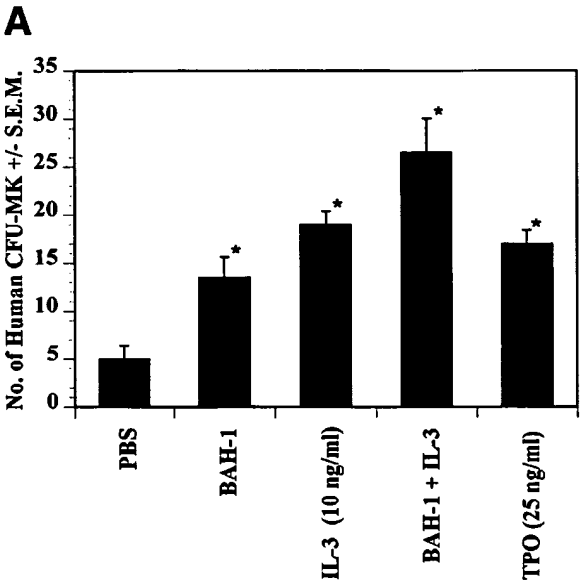
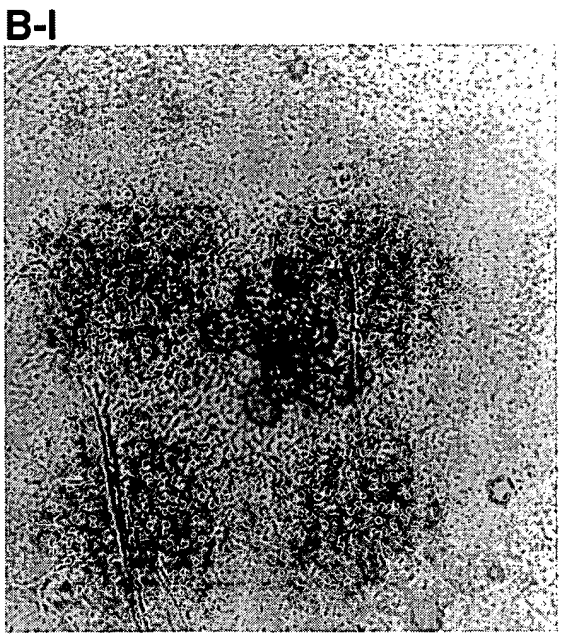
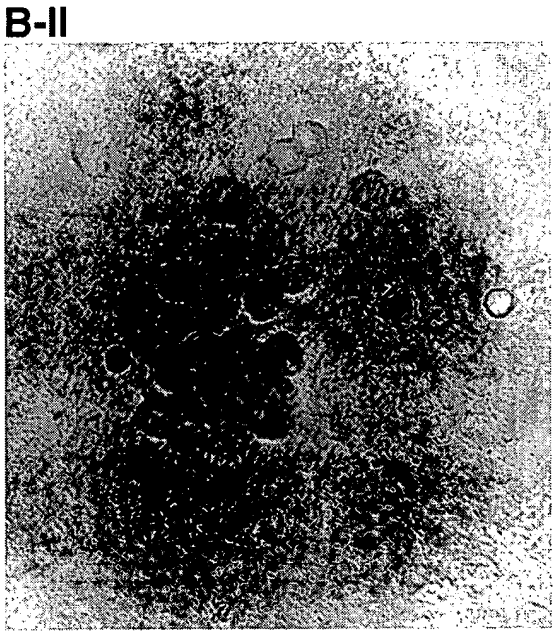



**Fig 5. Effect of BAH-1 MoAb on human CFU-MK colonies. (A) $CD34^+$ bone marrow cells were plated at $5 \times 10^3$/mL in the fibrin clot system (see the Materials and Methods). Results are expressed as the means ± SEM of megakaryocyte colonies. Experiments were performed in triplicate in four assays. *Statistically significant compared with PBS ($P < .05$). (B) Photographs of CFU-MK colonies in the presence of BAH-1 (B-I) or TPO (B-II).**



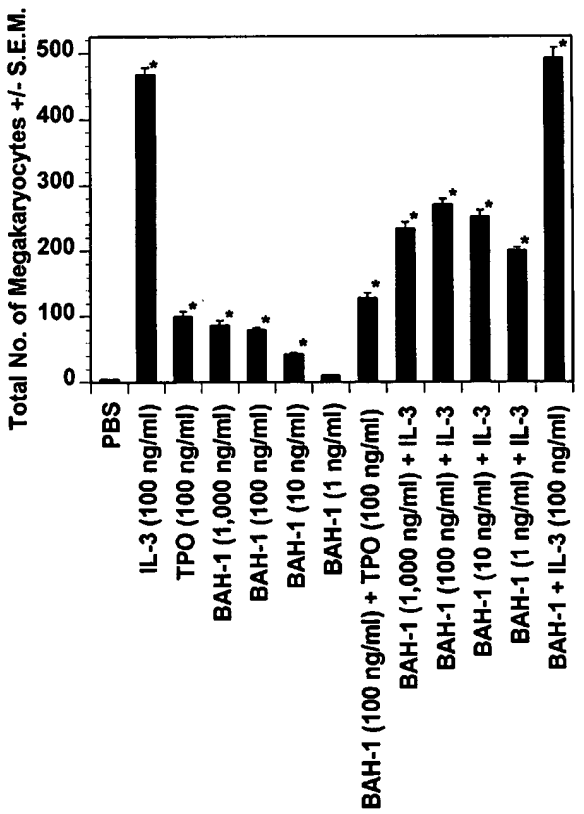



**Fig 6. Effect of BAH-1 on CD34⁺ CD41⁺ cells. CD34⁺ CD41⁺ cells were cultured under serum-free conditions in the presence of rhTPO, IL-3 (100 ng/mL), the optimal plateau concentration as determined for IL-3 with this assay, or various concentrations of BAH-1. On day 5, megakaryocytes in culture were quantitated visually by using an inverted microscope. *Statistically significant compared with PBS ($P < .05$).**

determined to be approximately 40% to 70%. Individual large megakaryocytes as well as small megakaryocytic cells were observed.

*Effect of BAH-1 on murine megakaryocytopoiesis.* Dot blot analysis indicated that BAH-1 also cross-reacts with murine Mpl (data not shown). Because of this, we determined whether BAH-1 was also an agonist for murine megakaryocytopoiesis. In a liquid suspension assay,[31] BAH-1 stimulated the proliferation of immature megakaryocytes (Fig 7). However, BAH-1 failed to stimulate murine CFU-MK colony formation (Fig 8) although it did appear to synergize with TPO or IL-3 in stimulating murine CFU-MK formation (Fig 8). These results suggest that BAH-1 does not induce complete differentiation of murine megakaryocytes.

*Effect of BAH-1 on hematopoiesis in myelosuppressed mice.* The effects of BAH-1 alone or in combination with TPO on hematopoietic progenitor cell numbers during the recovery phase after myelosuppressive therapy were evaluated in myelosuppressed mice. Only a modest increase in the numbers of CFU-MK colonies was observed in the marrow of BAH-1–treated mice (Table 1), whereas an increase in the numbers of CFU-MK colonies was observed in the BAH-1 plus TPO-treated mice (Table 1). No effects of BAH-1 were observed on

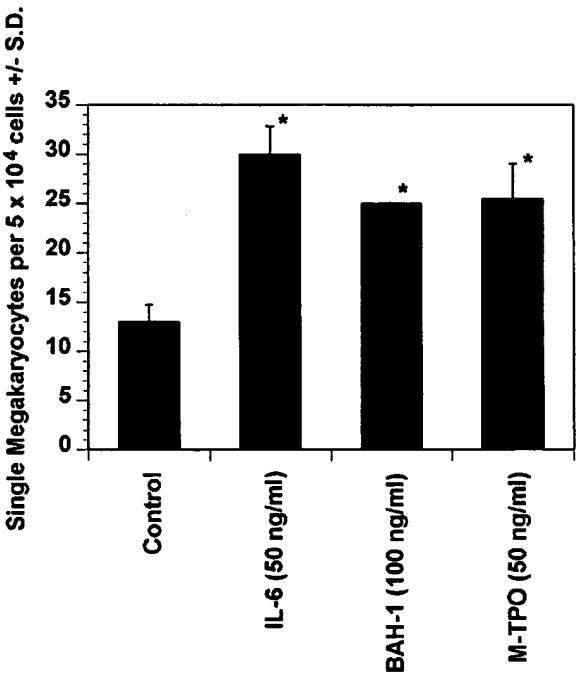



**Fig 7. Effect of BAH-1 MoAb on murine immature megakaryocytes by using a single megakaryocyte growth assay. Single megakaryocytes were scored as the number of acetylcholinesterase-positive cells per fractionated 5 × 10⁴ bone marrow cell cultures. Results are the means ± SEM from triplicate cultures from three experiments. *Results were significantly different ($P < .01$) from FCS control.**

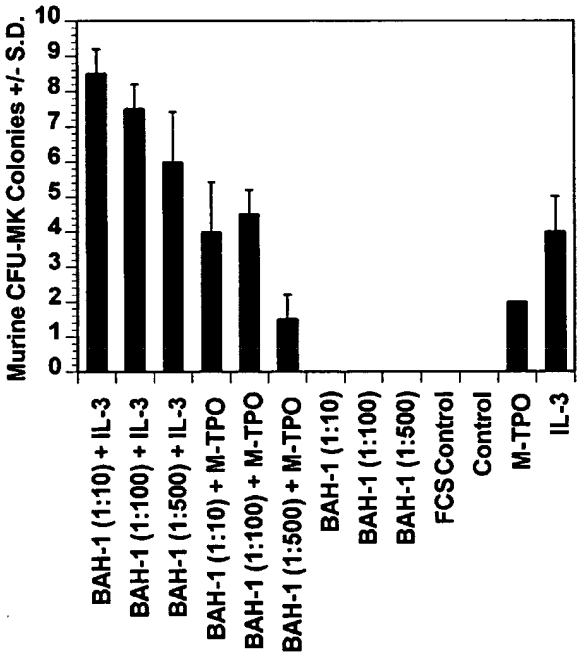



**Fig 8. Effect of BAH-1 MoAb on murine CFU-MK colonies. CFU-MK megakaryocytes were scored as the number of colonies with three cells or more per $10^5$ unfractionated cells. BAH-1 (1 μg/mL) was used in various dilutions as indicated. Results are the means ± SEM from triplicate cultures from three experiments. *Results were significantly different ($P < .01$) from FCS control.**

**Table 1. Marrow Hematopoietic Progenitor Cell Levels After the Administration of BAH-1, TPO, Control MoAb, or TPO plus BAH-1 to Myelosuppressed Mice**

| Treatment | CFU-MK ($\times 10^{-3}$) Experiment 1 | CFU-MK ($\times 10^{-3}$) Experiment 2 | CFU-MK ($\times 10^{-3}$) Experiment 3 | BFU-E ($\times 10^{-3}$) Experiment 1 | CFU-GM ($\times 10^{-3}$) Experiment 1 |
|---|---|---|---|---|---|
| Control | 0 | 0 | 0 | 2.0 ± 1.0 | 9.0 ± 1.0 |
| TPO | 10.5 ± 0.5* | 3.0 ± 1.0* | 7.5 ± 1.0* | 7.0 ± 1.0* | 50.5 ± 9.5* |
| BAH-1 | 1.0 ± 0 | 2.0 ± 1.0 | 2.0 ± 1.0 | 2.5 ± 1.0 | 12.0 ± 1.0 |
| BAH-1 + TPO | 12.0 ± 1.0* | 4.5 ± 0.5* | 12.5 ± 1.0* | 7.5 ± 0.5* | ND |

The results for femoral progenitors in each experiment represent the mean ± SEM of three animals in Experiments 1 and 2, whereas Experiment 3 included five animals in each treatment group.

Abbreviation: ND, not determined.

*$P < .005$ compared with control.

the CFU-GM, BFU-E, or CFU-E colonies (Table 1). The modest effect of BAH-1 in this model is likely because of the relatively modest effect of BAH-1 on murine megakaryocytopoiesis.

## DISCUSSION

We have generated a murine MoAb, BAH-1, against human megakaryocytic cells that specifically recognizes the TPO receptor, c-Mpl. BAH-1 specifically bound to platelets and recombinant c-Mpl with high affinity and had agonist activity in various assays of in vitro human and murine megakaryocytopoiesis, including the generation of CFU-MK megakaryocyte progenitors and the production of mature GpIIb/IIIa-expressing megakaryocytes in liquid cultures of heterogeneous bone marrow cells. Although BAH-1 was able to trigger cell proliferation and differentiation of human megakaryocytic precursors and immature murine megakaryocytes, by itself it failed to stimulate murine CFU-MK colony formation (Fig 7). However, it did synergize with IL-3 and TPO to stimulate murine CFU-MK formation. From these results, we conclude that BAH-1 is an agonist antibody specific for c-Mpl and is capable of stimulating human megakaryocyte growth and maturation.

TPO has an effect on stem cells as well as erythroid progenitors.[15,34] However, BAH-1 alone had no effects on BFU-E and CFU-E colonies under the conditions tested, as described.[15] The lack of potency of BAH-1 on the erythroid lineage could be caused by a cross-species effect, because BAH-1 is a murine antibody being tested in a human system. Alternatively, a cooperative effect with EPO could be needed to see an effect of BAH-1 on this lineage.

Homodimerization of the c-Mpl receptor by TPO on the cell surface is believed to be the key event in TPO-induced signal transduction.[17] In support of this, TPO has recently been shown to contain two receptor binding sites that function to dimerize c-Mpl.[37] Similarly, because of its bivalency, BAH-1 likely acts as an agonist by inducing homodimerization of c-Mpl. Indeed, monovalent Fab fragments of BAH-1 antibody, which cannot form receptor dimers, did not stimulate megakaryocytopoiesis (data not shown). These results suggest that the mechanism through which BAH-1 stimulates megakaryocytopoiesis is by "self-antagonism," which has been described for agonist antibodies for GH, PRL, and EPO receptors.[35,38,39]

BAH-1 offers several opportunities for improving our understanding of the biology of megakaryocytopoiesis as well as its potential therapeutic applications. More detailed mapping of the binding site of this MoAb on c-Mpl may help define the sequences and confirmation of the native receptor, which are necessary and sufficient for activation and subsequent signal transduction. Studies of different c-Mpl constructs are in progress to achieve this aim. It is interesting to note that BAH-1 does not antagonize TPO binding to c-Mpl, suggesting that they have different binding sites.

Currently, it is not known if BAH-1 stimulates platelet production in vivo. In a murine myelosuppressive model, BAH-1 only modestly affected megakaryocytopoiesis. This may be caused by species specificity because BAH-1 was much more effective in stimulating human versus murine megakaryocytopoiesis in vitro.

Although the most immediate clinical trials are of recombinant human TPO, there may be certain opportunities for the use of a humanized agonist MoAb. Humanizing murine antibodies have successfully yielded a number of products currently in clinical trials that have minimal antigenicity while sustaining affinity and functional potency in the recognition of human antigens. Because of the prolonged half-life of antibodies, it might be feasible to administer an agonist antibody to the c-Mpl receptor on an intermittent basis, thereby sustaining the stimulation of megakaryocytopoiesis in patients with a compromised production of cells of this lineage.

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